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(54) Title: TESTING FOR SPIROCHETAL NUCLEIC ACID SEQUENCES IN SAMPLES

(57) Abstract

Ticks, or other samples, are tested for the presence or absence of Lyme disease spirochete infestation by subjecting the sample to a polymerase chain reaction amplification for the detection of Borrelia burgdorferi spirochete nucleic acid sequences. Spirochete-specific primers and probes are used to perform the test.

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TESTING FOR SPIROCHETAL NUCLEIC ACID SEQUENCES IN SAMPLES

In the specification

This invention relates to a method of testing arthropod vectors, such as ticks, or other samples, for the presence or absence of Borrelia burgdorferi-related genetic material to determine whether the ticks are spreaders of Lyme disease.

Lyme disease has only recently been recognized by the medical profession as a distinct clinical entity. At present in the United States alone, more than six thousand new human incidences of Lyme disease occur each year. Lyme disease has been determined to be global in scope, and is not merely restricted to the Northeastern United States. Symptoms of Lyme disease may be slow to manifest themselves, and may not be diagnosed by even a skilled physician until after the infection has been present for many years.

At the present time, Lyme disease may only be diagnosed after telltale symptoms have manifested themselves in an infected patient. As noted above, this can entail a considerable period of time after initial infection, and thus can expose the patient to complications from the infection which are painful and even life threatening, in extreme cases.

Lyme disease is initiated in humans by bites from infected ticks. The most common source of Lyme disease, in humans, is the deer tick. Deer ticks which carry the Lyme disease-causing parasite spirochete, Borrelia burgdorferi, will infect humans with Lyme disease when bitten. As noted above, the presence of the Lyme spirochetes in a locale will not be realized until after one has been bitten by an infected tick; thus prevention of the disease, until an effective vaccine is developed, is not known to be an option.

This invention is directed to a procedure for detecting the presence or absence of Borrelia burgdorferi in the tick population. Following the precepts of this invention, ticks found in the locale can be tested on a regular basis so that, if infected ticks are noted, the local populace can take extra precautions against exposure to ticks. Ticks from bitten patients can also obviously be tested for the spirochete. It is a relatively simple procedure to gather ticks without exposing oneself to the danger of being bitten. Since ticks are attracted to changes in surrounding color and temperature, one merely needs to drag a white sheet or the like about one's yard and the sheet will pick up ticks, if there are any in the area. Additionally, if one, or one's pet is bitten by a tick, the tick can be tested after removal from the skin of the bitten subject.

The procedure of this invention can be used on dessicated tick bodies, whereby the ticks to be tested can be killed by placing them in a vial containing alcohol, wherein the ticks will be preserved, for subsequent testing.

In general, the procedure of this invention involves grinding or otherwise rupturing the tick body to release its midgut contents. The midgut contents are then subjected to a polymerase chain reaction amplification for the detection of the presence of Borrelia-specific nucleic acid sequences in the tick midgut contents. A positive result indicates that the subject tick is a carrier of the Lyme spirochete, while a negative result suggests the opposite.

Polymerase chain reaction (PCR) amplification of particular nucleic acid sequences in a sample is a procedure deriving from Cetus Corporation of Emeryville, California. U.S. Patents Nos. 4,683,195 to K. Mullis et al; and 4,683,202 to K. Mullis, both granted July 28, 1987, describe the PCR process, and are both incorporated herein in their entireties. PCR amplification is preferred to the use of conventional direct fluorescent antibody staining followed by microscopic examination of the tick material, because the PCR procedure can be used on alcohol-preserved, dessicated ticks. The fluorescent antibody procedure cannot be used on alcohol-preserved ticks because of high levels of background fluorescence.

I have developed certain new reagents for use in utilizing the PCR procedure to detect the Borrelia nucleic acid sequences in suspect ticks. My reagents for PCR amplification consist of oligonucleotide primers and probes encoding the OSP-A (outer surface protein) and flagellin proteins.

The oligonucleotide primers can be prepared on an Applied Biosystems 320A oligonucleotide synthesizer, desalted in a oligonucleotide purification cartridge, and used in the PCR reactions without further purification.

The sequences of the primers used for the OSP PCR amplification procedure (along with their optimum magnesium concentrations) are as follows:

the OSP-A gene target (1.75 mM $MgCl_2$):
OSP-A2: 5'GTT TTG TAA TTT CAA CTG CTG ACC 3'; and
OSP-A4: 5'CTG CAG CTT GGA ATT CAG GCA CTT C 3'; and
the probe
OSP-A3: 5'GCC ATT TGA GTC GTA TTG TTG TAC TG 3';
and the Flagellin gene target (1.75 mM $MgCl_2$):
FLA-1: 5'GAT GAT GCT GCT GGC ATG GGA GTT TCT GG 3'; and
FLA-3: 5'CTG TCT GCA TCT GAA TAT GTG CCG TTA CCT G 3';
and the probe
FLA-2: 5'ATT CAG ACA ACA GAA GGG AAT TTA GAA GTA G 3'.

For PCR analysis, tick specimens are air dried on filter paper disks for five minutes. Two hundred µl

(dry volume) of 0.5 mm glass beads are pre-treated in 1.0 ml of 1% bovine serum albumin in distilled water at 37 degrees C. for 30 minutes and then washed twice in 1.0 ml distilled water. The pre-treatment of the glass beads is necessary to prevent the beads from adsorbing the B. burgdorferi nucleic acid sequences during the PCR procedure. The ticks are placed whole into 0.6 ml microcentrifuge tubes containing 20 µl of a slurry of the pre-treated glass beads. The tick bodies are then crushed into the beads with a disposable plastic dowel for a time sufficient to liberate the midgut contents, and 25 µl of PCR buffer (10mM Tris HCL, pH 8.3; 50 mM KCL; 1.75 mM $MgCl_2$; 0.01% gelatin; 0.5% NP40 detergent; and 0.5% Tween 20 brand detergent) is added. The samples are then boiled for 5 minutes, and quenched immediately on ice, whereafter 5 µl aliquots of the treated samples are removed for amplification by the PCR procedure.

For amplification of Borrelia-specific sequences, 5 µl of the tick supernatant prepared as described above, is added to a 50 µl PCR reaction composition containing: 10 mM Tris HCL, pH 8.3; 50 mM KCL; 0.01% gelatin; 200 µm each deoxynucleotide triphosphate; and 50 picomoles of each primer, along with a concentration of $MgCl_2$ that is specific for each primer pair. All reactions can be performed in a Perkin Elmer-Cetus thermal cycler. The aforesaid components are denatured at 94 degrees C. for 30 seconds; annealed at 55 degrees C. for 45 seconds; and extended at 72 degrees C. for 1 minute, for a total of 45 cycles.

The amplification products are analyzed by electrophoresis in a 1% Seakem/3% Nusieve agarose gel, a product of FMC Bioproducts of Rockland, Maine, followed by staining with ethidium bromide and U.V. transillumination. The products are then blotted onto Hybond nylon membranes (a product of Amersham, of Arlington Heights, Illinois). The membranes are crosslinked on a 300 nm U.V. transilluminator for five minutes and prehybridized in a mixture of 5X. Denhardt's solution; 0.5% SDS; and 100 µg/ml denatured salmon sperm DNA at 55 degrees C. for one hour. Filters are hybridized, in the above solution with labelled oligonucleotide probe for 4-6 hours. After hybridization, the hybridized filters are exposed to Kodak XAR-5 film between intensifying screens for times ranging from one hour, at room temperature, to as long as four days at -70 degrees C. Two positive controls consisting of 1 ng of genomic B. burgdorferi DNA derived from the cloned strain N40; and five negative controls, with no DNA added, may be utilized in confirmation of the observed results.

The aforesaid procedure was performed on ticks that had recently fed on known positive and negative animals. The ticks were then processed as described above. Positive ticks were identified by the presence of a 146 base-pair OSP-A-specific amplification product which co-migrated with the positive controls. No such amplified species was observed among the known negatives.

The primary target for PCR detection of Borrelia burgdorferi sequences is the gene encoding the OSP-A of the reference strain B31. The gene for this protein resides on a 49 kb linear plasmid present in multiple copies per cell and it serves as a highly conserved target for the sensitive detection of most North American isolates of B. burgdorferi.

To verify the presence of Borrelia-specific DNA in infected specimens, a second primer set directed against the flagellin gene of strain B31 is used. This primer pair produces a 200 base-pair genus-specific amplification product. Borrelial species of many types, including the Borrelia hermsii and recurrentis, are detected with these primers, but not Treponema, leptospira or several exoflagellum-bearing organisms.

The specificity of the flagellin gene amplification products will be confirmed by slot-blot hybridization with an internal oligonucleotide probe (FLA-2) using the materials described herein.

It will be readily appreciated that the method of this invention, if applied to human or veterinary specimens, may greatly aid in diagnosing Lyme disease before symptoms appear, and thus can significantly limit the debilitating effects of the disease. The method can be performed with preserved tick specimens, which can be sent to remote laboratory for analysis.

While the procedure for detecting the specific spirochete DNA sequences has been described specifically for use on tick samples, it will be understood that the same procedure can be used to test human and animal specimens. The latter specimens can be cerebrospinal fluid, blood, urine, skin and synovial fluid.

As an example of the latter, DNA was extracted using standard procedures from synovial fluid specimens from patients suffering from arthritis. These samples all cultured negative for the Lyme spirochete using the prior art culture procedures; however, a number of the samples were found to contain spirochetal DNA by amplification OSP-A sequences, suggesting ongoing infection in these patients.

Since many changes and variations of the disclosed embodiment of the invention may be made without departing from the inventive concept, it is not intended to limit the invention otherwise than as required by the appended claims.

What is claimed is:

CLAIMS

1. A method of testing ticks for the presence or absence of Borrelia burgdorferi genetic material, comprising the steps of:
a) extracting the midgut contents of the ticks; and
b) performing a polymerase chain reaction amplification for the detection of Borrelia-specific nucleic acid sequences in said tick midgut contents.

2. The method of Claim 1 wherein said ticks are desiccated prior to being tested.

3. The method of Claim 2 wherein said ticks are preserved in alcohol prior to being tested.

4. A method of testing ticks for the presence or absence of Borrelia burgdorferi genetic material, said method comprising the steps of:
a) treating the ticks in such a manner so as to release the tick midgut contents for analysis; and
b) performing a polymerase chain reaction amplification for the detection of Borrelia-specific nucleic acid sequences in said midgut contents.

5. The method of Claim 4 comprising the further step of preserving the ticks in alcohol prior to performing the test.

6. A primer mixture for use in a nucleic acid amplification procedure for detecting the presence of Borrelia DNA material in a sample, said mixture comprising the OSP A gene primers with the following sequences:

OSP-A2: 5'GTT TTG TAA TTT CAA CTG CTG ACC 3'; and
OSP-A4: 5'CTG CAG CTT GGA ATT CAG GCA CTT C 3'; and
the Flagellin gene primer sequences:
FLA-1: 5'GAT GAT GCT GCT GGC ATG GGA GTT TCT GG 3'; and
FLA-3: 5'CTG TCT GCA TCT GAA TAT GTG CCG TTA CCT G 3'.

7. A probe for use in a nucleic acid amplification procedure for detecting the presence of Borrelia DNA material in a sample, said probe being:
OSP-A3: 5'GCC ATT TGA GTC GTA TTG TTG TAC TG 3'.

8. A probe for use in a nucleic acid amplification procedure for detecting the presence of Borrelia DNA material in a sample, said probe being:
FLA-2: 5'ATT CAG ACA ACA GAA GGG AAT TTA GAA GTA G 3'.

9. A method which can be performed individually, or as a step in an integrated reaction, for detecting Borrelia-specific nucleic acids in a sample, said method comprising the steps of: adding to the specimen a primer mixture for a first Borrelia gene; adding to the specimen a primer mixture for a second Borrelia gene; performing a polymerase chain reaction amplification on the sample; adding to the specimen a

first probe for detecting any amplified segments of said first Borrelia gene; and adding to the specimen a second probe for detecting any amplified segments of said second Borrelia gene.

10. The method of Claim 9 wherein said gene primer is an OSP-A gene primer.

11. The method of Claim 10 wherein said second gene primer is a Flagellin gene primer.

12. A method which can be performed individually, or as a step in an integrated reaction, for detecting Borrelia-specific nucleic acids in a sample, said method comprising the steps of: adding to the specimen a primer mixture for a Borrelia gene; performing a polymerase chain reaction amplification on the sample; and adding to the specimen a probe for detecting any amplified segments of said Borrelia gene.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/08737

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12Q 1/68; C12P 19/34; C07H 15/12; C12N 15/00
US CL : 435/6, 91; 536/27; 935/77,78

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/6, 91; 536/27; 935/77,78

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

APS, CAS ONLINE

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
x	JOURNAL OF CLINICAL MICROBIOLOGY, Volume 28, No. 3, issued March 1990, Persing et al, "Detection of Borrelia burgdorferi Infection in Ixodes dammini Ticks with the Polymerse Chain Reaction", pages 566-572, see entire document.	1-12

* Special categories of cited documents:[16]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
02 MARCH 1992	16 MAR 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	Mindy B. Fleisher

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